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**14. ABSTRACT**

The purpose of the proposed research is to develop novel optical technologies to identify high-risk premalignant changes in the breast. Our proposed research will first test specific optical parameters in breast cancer cell lines and models of early mammary carcinogenesis, and then develop methods to test the optical parameters in random periareolar fine needle aspirate (RPFNA) samples from women at high-risk for developing breast cancer. Over the last year, we have found that the optical redox ratio can 1) differentiate normal mammary epithelial cells from breast cancer cell lines, 2) differentiate ER(-) and ER(+) breast cancer cell lines and 3) monitor response to ER-targeted therapies. Our results suggest that the optical redox ratio will likely be able to differentiate normal mammary epithelial cells from premalignant changes. Further the optical redox ratio, in combination with other optical parameters, may be useful to monitor response to therapy, or predict response to therapy in patients with breast cancer and in small animal models.

**15. SUBJECT TERMS**
Optical spectroscopy, RPFNA, optical redox ratio, Estrogen Receptor

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## INTRODUCTION

Progress in breast cancer prevention is currently limited by *1)* our inability to detect pre-invasive breast cancer and *2)* a lack of biological markers to risk-stratify mammary atypia. Optical Spectroscopy (auto fluorescence, absorption, and scattering) is a powerful experimental imaging technique that is currently being developed to exploit differences in the vascularity, hemoglobin oxygenation, fluorescent amino acids, metabolic proteins and structural proteins in normal and high-risk breast tissues. My mentor, *Dr. Nimmi Ramanujam* has shown that optical spectroscopy can differentiate metabolic differences between normal breast tissue from invasive breast cancer. The overall goal of our current research is to determine if Optical Spectroscopy can detect high-risk pre-invasive breast changes from normal breast tissue. We have taken a targeted approach, first specifically testing the optical redox ratio, which can be imaged by confocal microscopy. We have optimized the optical parameters in normal mammary epithelial cells and breast cancer cell lines and will move on to testing the optical redox ratio from epithelial cells obtained by Random Periareolar Fine Needle Aspiration (RPFNA). RPFNA is a research technique designed to prospectively sample breast cytology from asymptomatic high-risk women. My mentor, Dr. Victoria Seewaldt, has used RPFNA to test for pre-malignant cytological changes in over 250 high-risk women. In this multi-disciplinary postdoctoral fellowship, I will integrate the technology developed by my two mentors, to test whether the Optical Spectroscopy, can be used to improve the ability of RPFNA to predict short-term breast cancer risk.

## BODY

The first task outlined in the Statement of Work (SOW) was to "Acquire approval from the Department of Defense for research on samples from human subjects." This task has been accomplished and the proposed project has been approved by both the DoD and Duke University.

The second task was "To test UV-Visible fluorescence signatures in defined *in vitro* models of early mammary carcinogenesis." Based on our findings presented in the preliminary data section of the proposal (Figure 1), we first examined the optical redox ratio (NADH/FAD) on a broader panel of breast cancer cell lines. We quickly realized that in addition to cells segregating based on retinoid sensitivity, the optical redox ratio also differentiated cell lines based on expression of the estrogen receptor (ER). To date we have tested the optical redox ratio in a combined total of 11 normal mammary epithelial (2), ER(-) breast cancer (4) and ER(+) breast cancer (5) cell lines (Figure 2).
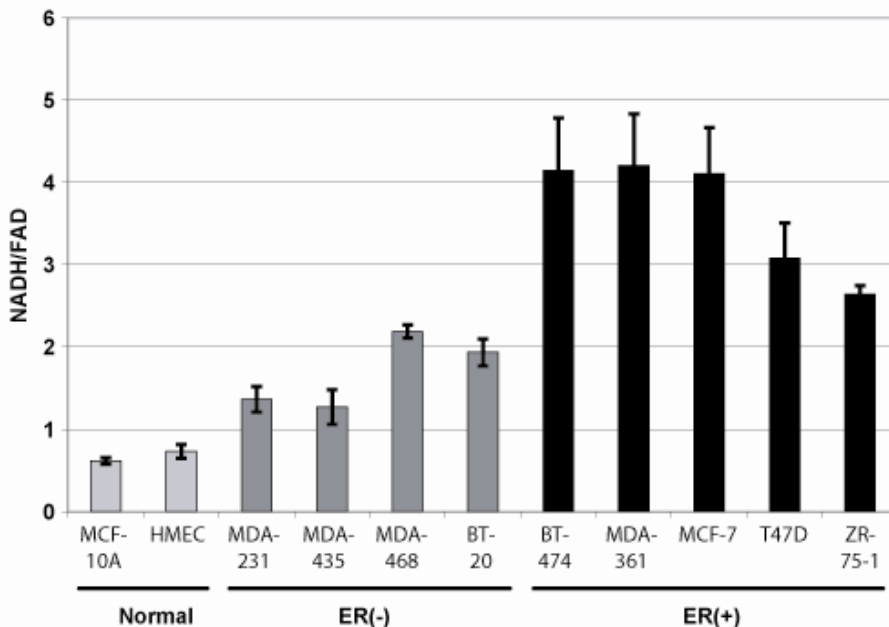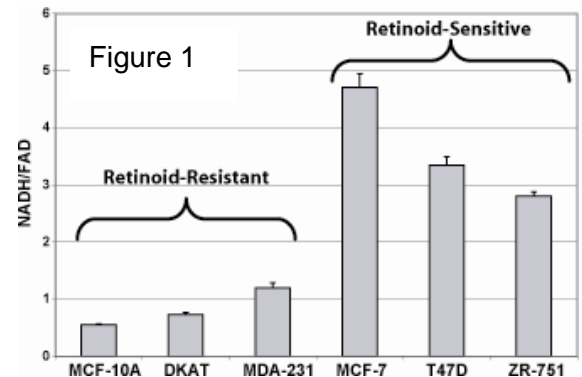
**Figure 2.** Optical Redox Ratio of breast cancer cell lines acquired using a Zeiss 410 laser scanning confocal microscope. NADH intensity images were obtained by excitation at 351 nm and collecting the fluorescence emission with a 400 nm LP filter. FAD intensity images were obtained by excitation at 488 nm and collecting the fluorescence emission with a 505 nm LP filter. At least 8 images were analyzed for each cell line. Every cell per image was analyzed separately. The optical redox ratio from all cells in an image was averaged, and the data from one image represented one data point (n = 1). Error bars represent standard error.

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From this panel of cell lines we can make a number of conclusions. First, we found that all breast cancer cell lines have a statistically significant, by pairwise student's t-test, higher optical redox ratio compared to normal mammary epithelial cells. Second, if cell line data is grouped based on 1) normal mammary epithelial cells, 2) ER(-) breast cancer cells and 3) ER(+) breast cancer cells, all groups are statistically different from each other (student's t-test p < 0.05). This means that ER(+) cells have a higher redox ratio than ER(-) and normal mammary epithelial cells, and ER(-) cell lines have a higher redox ratio than normal mammary epithelial cells, but lower than ER(+) breast cancer cell lines (Figure 3).
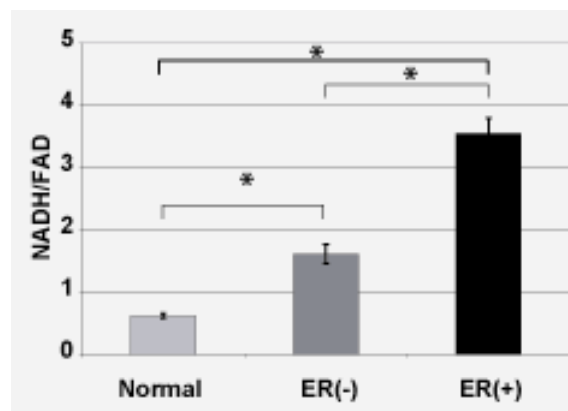


**Figure 3.** Data from cell lines shown in Figure 2 was combined based on ER receptor expression. Error bars represent standard error. Asterisk (*) p < 0.0001.

The cell line data suggests that expression of ER increases the optical redox ratio. To determine if ER expression correlates with the optical redox ratio, we performed quantitative real-time PCR on cDNA prepared from RNA isolated from each of the cell lines. As shown in Figure 4, all ER(+) cells express ESR1 at high levels (at least 4 fold higher than ER(-) cell lines). A Pearson correlation coefficient was calculated to determine the linear relationship between the optical redox ratio and ESR1 expression levels and found to be significant (p = 0.0024, r = 0.81), further suggesting that ER expression has an effect on the optical redox ratio.
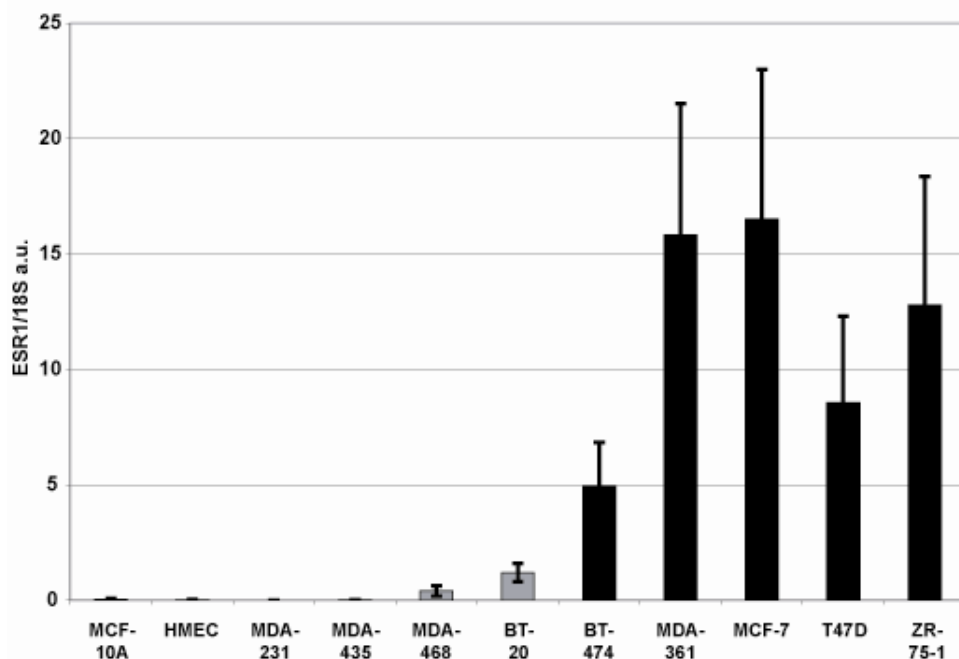


**Figure 4.** Total RNA was isolated from all cell lines and cDNA prepared. Quantitative real-time PCR (qRT-PCR) was then performed on the cDNA. All qRT-PCR reactions were performed in triplicate. ESR1 levels were normalized to qRT-PCR reactions for 18S rRNA. Figure 4 is the average of four separate experiments. Error bars represent standard deviation.

Next, we performed a series of experiments to determine if inhibition of ER function has an effect on the optical redox ratio. First, ER function was inhibited with tamoxifen (Tam), an ER antagonist in the breast. Tam is known to promote cell cycle arrest and apoptosis in ER(+) breast cancer and is frequently prescribed to women with ER(+) breast cancer. Tam is also an effective chemoprevention drug for women at high-risk for developing breast cancer [1]. ER(+) MCF-7 and T47D cells were treated with 2 μM tamoxifen (Tam) for 48 hours and the optical redox ratio was measured by confocal microscopy. We found that Tam significantly decreased the optical redox ratio of both MCF-7 and T47D breast cancer cells (Figure 5A). In a similar set of experiments we treated ER(+) MCF-7 cells and ER(-) MDA-231 cells with Tam and the pure antiestrogen ICI 182, 780 (ICI, Faslodex). As expected, Tam and ICI significantly reduced the optical redox ratio of MCF-7 cells, but had no effect on MDA-231 breast cancer cells (Figure 5b). We also acquired MCF-7 variant cell lines

LCC2 and LCC9 from Robert Clarke at Georgetown University [2]. LCC2 cells are Tam-resistant and ICI-sensitive, while the LCC9 cells are Tam- and ICI-resistant. Parental MCF-7, LCC2 and LCC9 cells were either left untreated or treated with ICI for 48 hours. ICI had a statistically significant effect on MCF-7 and LCC2 cells (student's t-test, $p < 0.05$), while the LCC9 cells were not effected by the ICI treatment (Figure 5c). Together, the data in Figure 5 suggest that ER antagonists reduce the optical redox ratio in ER(+) breast cancer cells, but not ER(-) or resistant cell lines.
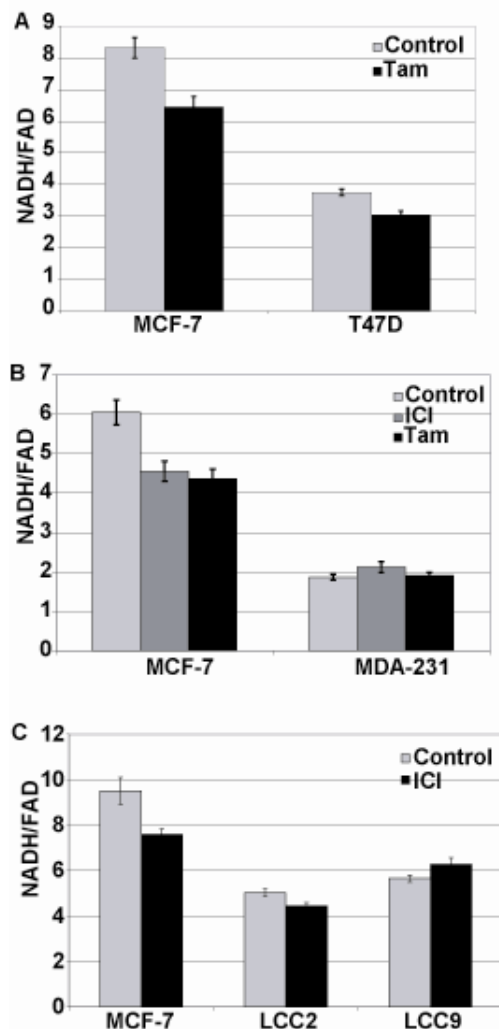


**Figure 5. A,** Optical redox ratio was measured by confocal microscopy from ER(+) MCF-7 and T47D cells that were treated with 2 $\mu$M tamoxifen (Tam) for 48 hours. **B,** Optical redox ratio was measured from control-, ICI-182,780- (ICI), and Tam-treated ER(+) MCF-7 and ER(-) MDA-231 cells. **C,** Optical redox ratio was measured from MCF-7 derived LCC2 (Tam-resistant, ICI-sensitive) and LCC9 (Tam-resistant, ICI-resistant), and MCF-7 cells treated with ICI for 48 hours.

## KEY RESEARCH ACCOMPLISHMENTS

- We have shown that the optical redox ratio can clearly differentiate normal mammary epithelial cells from breast cancer cell lines.

- The optical redox ratio can differentiate ER(+) from ER(-) breast cancer cell lines.

- The optical redox ratio is modulated by ER expression and is reduced in the presence of ER antagonists.

## REPORTABLE OUTCOMES

- Ostrander JH, et al. Optical Redox Ratio Differentiates Breast Cancer Cell Lines Based on Estrogen Receptor Status. Endocrine Society Meeting, San Francisco, CA; June 2008, Poster Presentation.

- Ostrander JH, et al. Optical Redox Ratio Differentiates Breast Cancer Cell Lines Based on Estrogen Receptor Status. DoD LINKs Meeting, Vienna, VA; February 2009, Poster Presentation.

The research described above was used as preliminary data for the submission of two grant applications in collaboration with Dr. Nimmi Ramanujam.

- DoD Era of Hope – BC087569. Harnessing the power of light to see and treat breast cancer.  The goal of this proposal is to design and develop novel optical strategies to make current treatments for breast cancer faster and more effective, thereby reducing over- or under-treatment and the time and cost burden associated with it. To achieve this, we will leverage optically detectable biomarkers that report on the physiological, metabolic, molecular and morphological state of the cancer, with novel technologies being developed by our team.

- NIH R01 – AN:3146201. Multiparametric biomarker approach to asses tumor response to therapy.  The goal of this R01 proposal is to develop a non-invasive, multi-parametric optical biomarker platform to dynamically characterize tumor hypoxia and angiogenesis in small animal models

- The data presented above is also being prepared for submission to Cancer Research.


## CONCLUSION

Our results suggest that the optical redox ratio can 1) differentiate normal mammary epithelial cells from breast cancer cell lines, 2) differentiate ER(-) and ER(+) breast cancer cell lines and 3) monitor response to ER-targeted therapies.  These studies provide further evidence that the optical redox ratio may identify premalignant changes from high-risk women.  We will continue to develop models of mammary atypia and use our current methodologies to test our hypothesis in vitro.  We are also developing new methodologies to test our hypothesis in samples from women at high-risk for developing breast cancer.  Furthermore, these studies suggest that optical techniques may allow us to quickly determine if a woman is responding the chemoprevention therapies, such as tamoxifen.

While our results support the proposed research, there are also implications beyond the scope of the funded proposal.  First, the optical redox ratio, in combination with additional optical parameters (total hemoglobin, hemoglobin saturation, and scattering), may have the power to assess the physiological, metabolic, morphological and molecular alterations in breast tissue in response to targeted and chemotherapies.  To further investigate this possibility we (Dr. Ramanujam, Dr. Ostrander and others) recently submitted an Era of Hope proposal to the DoD BCRP.

Second, it is possible that Optical Spectroscopy and other molecular imaging modalities (such as optical coherence tomography (OCT)) when combined will have the power to quickly and efficiently monitor response to therapy and allow to further probe the molecular mechanisms associated with chemotherapy resistance.  We have recently submitted an R01 proposal in which we propose to develop a non-invasive, multi-parametric optical biomarker platform to dynamically characterize tumor hypoxia and angiogenesis in small animal models. The system developed through these proposed studies will be a powerful tool for studies on mechanisms of tumor growth, resistance and response to therapy


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